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Ki-67 Expression in Human Tumors Measured by Flow Cytometry

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Ki-67 EXPRESSION IN HUMAN TUMORS MEASURED BY FLOW CYTOMETRY

By

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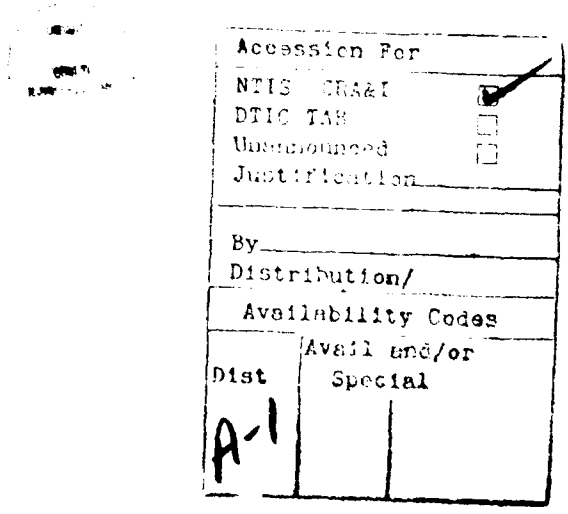

Abstract of Thesis Presented to the Graduate School
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Kinetic information is important in assessing tumor growth because it provides significant prognostic and therapeutic information. Methods for clinical kinetic analysis include ^{3}H-thymidine labelling, DNA analysis by flow cytometry (FCM), and immunohistochemical detection of cell cycle dependent antigens. DNA analysis by FCM is most widely used because the method is fast, cells can be stained easily, the analysis is automated, and the results are numerical and objective. However, simple DNA analysis is somewhat limited since it provides only partial information on the cell cycle phases and is subject to certain artifacts such as interference by nuclear debris or cell clumps.

This study sought to combine FCM analysis of DNA with a proliferation-associated antigen widely used in immunocytology, Ki-67. This antigen is expressed in the nucleus of proliferating cells throughout the cell cycle, but not

in nonproliferating cells. Existing methods for simultaneous staining of DNA and Ki-67 are unreliable. Therefore, our efforts were directed to developing a satisfactory staining technique. A short fixation with 0.5% paraformaldehyde followed by exposure of cells to 0.1% Triton X-100 was found to be effective. Ki-67 expression in human tumors measured by FCM was then compared with immunocytologic measurements and to the S-phase fraction calculated by DNA analysis only. A significant correlation was found between Ki-67 expression determined by immunocytology and FCM in non-Hodgkin's lymphoma (NHL) ($r=0.91$). Significant correlation was also found between Ki-67 expression determined by FCM and DNA S-phase in these tumors ($r=0.95$). An interesting finding was the detection of low levels of Ki-67 in nonproliferating cells using FCM. This low level expression is not detectable by immunocytology, a technique less sensitive than FCM.

In contrast to NHL, no significant correlation was found between Ki-67 expression and DNA S-phase in solid tumors such as colon cancers ($r=0.53$) or breast cancers ($r=0.19$). These tumors are difficult to disaggregate into single cell suspensions and the lack of correlation may result from leakage of the antigen from the cell nucleus as a result of cell dissociation, or even to artifactual errors in the cell cycle calculations. Although pathologists use empirical Ki-67 expression as an indicator of tumor proliferation, better antigenic characterization and further technological developments in cell preparation may be necessary before quantitative FCM analysis of this antigen can be applied universally to solid tumors.

INTRODUCTION

Cell Cycle Characteristics

The general biological model used to describe proliferating cells was introduced by Howard and Pelc (1953). This model was based on DNA content and divided the growth of proliferating cells into a cell cycle with four phases: G1, S, G2, and M. Nonproliferating diploid cells all had the same DNA content and were said to be in the non-growth or G0 phase of the cell cycle. In proliferating cells, the DNA content remains at the same amount as nonproliferating cells between the initiation of cell proliferation and the onset of DNA synthesis, an interval defined as the first growth or G1 phase. DNA content increases until it has doubled in the DNA synthesis or S-phase of the cycle. DNA content then remains constant at the doubled amount during the subsequent second growth or G2 phase and during the mitosis or M-phase. At the completion of the cell cycle the original cell has been replaced by two cells each with a DNA content of a nonproliferating diploid cell. The S-phase was confirmed by pulse labelling of growing cells using radiolabelled DNA precursors, particularly ^{3}H-thymidine, in a study by Cleaver (1967).

During the past 10 years, flow cytometry has been the most frequently used technique for studying the cell cycle. To obtain a DNA histogram using

this technique, a single cell suspension of the cells to be analyzed is prepared. The cells are then permeabilized using a detergent solution or an appropriate fixation technique, and stained with a DNA specific fluorescent dye. The labelled cells are aspirated into the flow cytometer where they are forced to flow as single cells through a laser beam that excites the fluorescent dye. The fluorescence emitted is collected by a lens system, passed through a filter allowing only the desired wavelength to pass, measured by a photomultiplier tube, and the intensity recorded. The recorded fluorescence is proportional to the amount of DNA present in the cell. A computer program then plots the number of cells with the same fluorescent intensity against the range of fluorescent intensities measured to produce the DNA histogram.

One type of critical information sought from DNA histograms is the proliferative fraction or percentage of cells in S-phase. Information about the percentage of S-phase cells is critical because it can be predictive of the biologic behavior of a tumor. Determination of the percentage of cells in S-phase is typically obtained by partitioning DNA histograms into appropriate segments and counting the number of cells in each segment. However, there is no general method for assigning mixed cell populations with differing DNA contents to phases of the cell cycle based completely on the analysis of a DNA histogram.

Another type of critical information sought from DNA histograms is the presence or not of aneuploidy, a term used to describe a sample containing a stem line with abnormal DNA content. Aneuploid cell populations are identified when two or more discernible peaks representing the normal and aneuploid cells

are present in a DNA histogram. Information about aneuploidy is also critical because it too can be predictive of the biological behavior of a tumor.

Tumor Cell Cycle Properties

Tumors are characterized by uncontrolled growth with differing degrees of cell proliferation. In most cases, tumors having the worst prognosis and poorest long term response to therapy are those with a high proliferation rate. For this reason, clinical stage and histologic grade are important prognostic criteria for most malignant tumors; however, survival remains mostly unpredictable in the individual patient using these criteria. In an attempt to develop additional prognostic criteria, many investigators have studied the relationship between *DNA S-phase and survival in patients with cancer.*

Most of the original studies of the DNA S-phase were done on non-Hodgkin's lymphoma (NHL), because intact lymphoma cells can be readily disaggregated into single cell suspensions by simple mechanical disruption. With improvement in techniques in recent years, the measurement of DNA content in other solid tumors by flow cytometry has also become accepted as a valuable procedure of diagnostic pathology. As with NHL, the DNA histograms for solid tumor cells can contain significant diagnostic or prognostic information and may influence therapeutic decisions. A review of the literature on the relationship between measurements of the DNA S-phase of solid tumors by flow cytometry and patient survival was done by Seckinger *et al.* (1989). They reported a significant increase in survival for patients with a low proliferative activity instead

of a high proliferative activity, as determined by the S-phase, in breast and lung tumors. Similar correlations have been found repeatedly in lymphomas. Studies of the DNA S-phase in other tumor types have varied in conclusions on survival.

For the calculation of the S-phase in tumors, DNA histograms are classified into three types: diploid, aneuploid with clear separation of diploid and aneuploid cells, and aneuploid with overlap of diploid and aneuploid populations. Cell suspensions obtained from most solid tumors contain less than 75% tumor cells (Frankfurt *et al.*, 1984). The remaining cells consist mainly of nonproliferating leukocytes and stromal cells. Thus, the S-phase DNA in diploid tumors reflects the proportion of tumor cell S-phase among mixed populations of nonproliferating or slowly proliferating normal cells. In two diploid tumors with similar proliferation activity, the S-phase will be lower for the tumor with a higher proportion of normal cells. Artificially low S-phases in diploid tumors caused by the presence of normal cells may be responsible for the lower value of S-phases in diploid tumors as compared with aneuploid tumors (Frankfurt *et al.*, 1984). Clear separation of diploid and aneuploid peaks, from which precise S-phase fractions could be calculated for the tumor population, were observed in less than 40% of 656 solid tumors examined (Frankfurt *et al.*, 1984). Problems in DNA S-phase calculation are also encountered in tetraploid tumors because of the presence of overlapping diploid G2/M cells with tetraploid G1 cells. A further complication of DNA analysis is different individual neoplastic cell populations within the tumor may have differing durations of the phases of the cell cycle. To achieve precision in the calculation of the DNA S-phase in

diploid, near-diploid, or tetraploid tumors in mixed populations, the ideal approach is to apply multiparameter flow cytometry and measure tumor or cell proliferation antigens simultaneously with DNA content. By measuring both parameters, more detailed and accurate information can be obtained, facilitating DNA analysis.

Determination of Proliferating Cells

Studies have indicated that cellular proliferation can be of prognostic significance. In particular, tumors of similar histological types often have shown a wide range of cell proliferation and studies show patients having tumors with high proliferative indices have a worse clinical course than those with low indices (Kvaloy *et al.*, 1981; Kvaloy *et al.*, 1985; Roos *et al.*, 1985; Schrape *et al.*, 1987). For the detection of proliferating cells, approaches used include: mitotic index evaluation, *in vitro* or *in vivo* ^{3}H-thymidine labelling index (Steel, 1977), primer-dependent DNA polymerase (Nelson & Schiffer, 1973), flow cytometric S-phase fraction, and the more recent monoclonal antibodies against bromodeoxyuridine (BrdUrd) and proliferating cell nuclear antigen (PCNA).

The identification of proliferating cells originally required the assessment of the number of mitoses in histological sections. This method has the specific disadvantages that mitosis is only part of the cell cycle, mitoses may continue through to interphase if tissues are not promptly fixed (Graem and Helweg-Larsen, 1979), and quantitation of mitotic figures in paraffin or other histological material can be unreliable. General disadvantages of this method are that the

interpretation of histological sections has suffered from poor reproducibility (Berard, 1985) and may lack the sensitivity to detect minimal disease (Weinberg *et al.*, 1984; Stetlet-Stevenson *et al.*, 1988).

Because of these disadvantages, a method more objective than microscopy has been sought. A variety of methods for the analysis of tumor cell proliferation were used to study the relationship between proliferation and histological grade in NHL (Silvestrini *et al.*, 1977; Braylan *et al.*, 1980; Kvaloy *et al.*, 1985). Emerging as the predominant techniques were ^{3}H-thymidine incorporation and flow cytometry. With regard to microscopy, both techniques were numerical, more reproducible, and more objective. Peckham and Cooper (1970) performed one of the earliest studies of NHL utilizing ^{3}H-thymidine and found no correlation between labelling index and response to treatment or survival. More recently Kvaloy *et al.* (1985) used B-cell lymphomas to determine DNA synthesis by ^{3}H-thymidine uptake and found uptake was greater among high grade than low grade specimens. Even among patients with low grade specimens, patients with specimens having higher uptake had shorter survival than patients with specimens having lower uptake. Still, ^{3}H-thymidine incorporation had its disadvantages including: its use of radioactivity, its requirement for *in vitro* incorporation, and it is labor and time intense.

In comparison with ^{3}H-thymidine incorporation, flow cytometry is fast, labelling easy, analysis more automated, and dual labelling can be done. DNA analysis using flow cytometry has been applied to NHL more than any other type of human tumor, principally because of the ease of tumor disaggregation.

Studies by Braylan *et al.* (1978) and Diamond and Braylan (1980) showed a correlation between the S-phase cells present and histological type of NHL with more S-phase cells present in high grade than in low grade tumors. Braylan *et al.* (1980) and Silvestrini *et al.* (1988) both showed a correlation between the DNA S-phase and ^{3}H-thymidine uptake. In the study by Braylan *et al.*, patients with greater than a 5% S-phase had a significantly worse survival than those with a S-phase less than 5%. Using histological grading of tumors as the standard for comparison, DNA S-phase was shown to correlate well by Hansen *et al.* (1981) and Braylan *et al.* (1984). Thus, DNA analysis using flow cytometry was confirmed as supplying useful prognostic information. Still, the limitations of flow cytometry in DNA analysis must be considered. The S-phase cannot always be evaluated when the tumor is aneuploid or tetraploid. Similarly, S-phase size alone gives only a partial picture of tumor kinetics. Kinetic parameters, including the duration of the cell cycle and of its phases, may be important.

With the large increase in available techniques in immunohistochemistry of recent years, interest has been focused on cellular antigens that reflect cellular proliferation. One of the first proliferation antigens to be studied was the transferrin receptor (TfR). Proliferating normal and tumor cells require iron and it could be expected they would express receptors for transferrin. A TfR binding monoclonal antibody was found by Trowbridge and Omary (1981). Since it seemed likely the numbers of TfR positive cells in a tumor would be related to its growth fraction, Habeshaw *et al.* (1983) applied a monoclonal antibody to cell suspensions of high and low grade NHL and showed a correlation between

TfR positive cells and histological grade. However, subsequent studies of expression of the TfR by Schrape *et al.* (1987) and Pileri *et al.* (1987) both concluded the expression of this receptor is not exclusive to proliferating cells. Since then, research using this antigen for detection of proliferation has declined.

A monoclonal antibody to BrdUrd was described by Gratzner (1982). Cells exposed to BrdUrd incorporate it into their DNA when replicating and the antibody can be used to detect incorporation. Using flow cytometry, it was found the binding of the antibody was equivalent to results obtained in ^{3}H-thymidine uptake studies and the fluorescent intensity was proportional to the amount of BrdUrd bound in each cell (Silvestrini *et al.*, 1988). Schrape *et al.* (1987) used anti-BrdUrd on high and low grade NHL and showed high grade lymphomas had more labelled cells than did low grade ones. Limitations in the use of BrdUrd for the determination of proliferating cells include: fluorescent intensity of the bound antibody is extremely dependent on the procedure used to denature the DNA to expose the incorporated BrdUrd, cell losses resulting from the denaturation procedure, the safety of *in vivo* injection of BrdUrd for the most effective labelling, and the inability of BrdUrd to incorporate after as little as overnight refrigeration (personal observation).

Miyachi *et al.* (1978) detected autoantibodies in patients with systemic lupus erythematosus that reacted with the nuclei of mitogen-stimulated human lymphocytes and proliferating cell lines. Two studies showed the antigen detected was PCNA, an acidic polypeptide with a molecular weight of about 36 Kilodaltons (Miyachi *et al.*, 1978; Celis *et al.*, 1984). PCNA has been shown to

be identical to another nuclear proliferation antigen called cyclin (Takasaki *et al.*, 1984; Ogata *et al.*, 1985). The principal limitation of the use of PCNA for the determination of proliferating cells is its peak expression being limited to the S-phase of the cell cycle. This S-phase association has been shown for both the autoantibody and a monoclonal antibody produced (Kurki *et al.*, 1986; Kurki *et al.*, 1988). Also, PCNA cannot separate G0 cells from G1 cells or G2 cells from M-phase cells (Landberg *et al.*, 1990).

Monoclonal Antibody Ki-67

Of the immunohistochemical techniques for investigating proliferating cells, the most commonly used procedure involves an antibody produced in 1983 and designated 'Ki-67' (Gerdes *et al.*, 1983). Ki-67 was originally produced against a crude nuclear fraction of the L428 cell line derived from Hodgkin's disease tissue. The study was aimed at producing monoclonal antibodies specific to Hodgkin and Sternberg-Reed cells by injection into mice of L428 nuclei obtained by incubation in NP-40. When the antibody was used to stain frozen sections of human tonsil, the chromosomes were stained. The antibody was further characterized by means of a differentiation-inducing stimulant applied to K562 erythroleukemic cells and peripheral blood lymphocytes after mitogen stimulation (Gerdes *et al.*, 1984b). This study used DNA analysis by flow cytometry to follow the expression of the Ki-67 antigen through the cell cycle, and found Ki-67 was present in the G1, S, G2, and M phases, but absent in G0.

Evidence supports the antibody recognizing the Ki-67 antigen can be used as a marker of cellular proliferation. First, Ki-67 immunoreactivity was present in cells that appear to be proliferating (Gerdes *et al.*, 1983). Second, the induction of antigen expression in mitogen-triggered peripheral blood lymphocytes and loss of the antigen on induction of differentiation in phorbol ester-treated HL60 cells (Gerdes *et al.*, 1984b). Third, a close relationship between the degree of expression of the antigen and phases of the cell cycle (Gerdes *et al.*, 1984b; Schwarting *et al.*, 1986). Fourth, a good correlation was found between Ki-67 immunocytology and uptake of BrdUrd (Schrape *et al.*, 1987).

The nature of the nuclear proliferation antigen that reacts with Ki-67 remains unknown. Nuclei, nucleoli, and chromosomes in metaphase are well outlined with this antibody, with preferential staining of nucleoli and condensed chromatin (Sasaki *et al.*, 1987). Demonstration of the localization of Ki-67 by electron microscopy has not been done, nor has it been identified by Western blot or immunoprecipitation assays, probably because the antigen appears to be disturbed by the usual fixatives and experimental procedures. The antigen is known to be sensitive to DNAse I, but not RNase, thus indicating the antigen has DNA binding properties (Sasaki *et al.*, 1987). In addition, the antigen has been shown to have inhibitory activity against DNA polymerase α (Loke *et al.*, 1987). Similarities have been observed in nuclear localization by immunocytology between Ki-67 and topoisomerase II, and although the similarities are not sufficient to conclude these two proteins are the same,

neither do any differences exist to conclude they are different (Bading *et al.*, 1989; Verheijen *et al.*, 1989).

The potential value of Ki-67 in the assessment of lymphomas was shown in a study by Gerdes *et al.* (1984a). They showed 93.8% of the high grade and 88.5% of the low grade specimens evaluated could be assigned accurately to their histological groups, according to the Kiel convention, on the basis of their counts of Ki-67 positive cells. Reports also indicate Ki-67 immunocytology gives cell proliferation data in NHL comparable to results of other methods (Gerdes *et al.*, 1984a; Schrape *et al.*, 1987). The range and median values of Ki-67 for all histological groupings, including low and high grade lymphomas taken together, were very similar in the studies of both Gerdes *et al.* (1984a) and Schrape *et al.* (1987), indicating the reproducibility of Ki-67 immunocytology.

Ki-67 expression has been used to assess tumor cell proliferation in breast tumors (Gerdes *et al.*, 1986; Barnard *et al.*, 1987), colorectal tumors (Horny and Horst, 1988), lung tumors (Gatter *et al.*, 1986), and intracerebral tumors (Burger *et al.*, 1986; Robson *et al.*, 1987). These studies demonstrate Ki-67 immunocytology is a useful means of measuring solid tumor growth fractions.

Several reports in the literature compared Ki-67 staining with other techniques for determining cellular proliferation. When applied to frozen sections of human tonsil, the antibody appeared to be reactive with a mitotic spindle-associated protein. It bound strongly to centroblasts, to a lesser extent to centrocytes, and variably to mantle zone lymphocytes. This corresponded with the findings for ^{3}H-thymidine uptake in tonsil tissue in the same study (Gerdes *et*

al., 1983). Another study evaluating Ki-67 expression in the monocytic/histiocyte cell line U937 and a concanavalin-A stimulated lymphocyte culture and also demonstrated a good correlation between Ki-67 expression and ^{3}H-thymidine uptake (Schwarting *et al.*, 1986). Schwartz *et al.* (1989) compared Ki-67 expression measured by immunocytology to DNA content in 74 cases of NHL and found a positive correlation between these two methods. Schrape *et al.* (1987) compared Ki-67 and TfR labelling in frozen sections of NHL and BrdUrd uptake in cell suspensions of NHL and showed TfR positive cells were numerous in low grade NHL regardless of histological type, but the numbers of Ki-67 positive cells correlated well with histological grade. An approach used by Sasaki *et al.* (1988) was to stain with both Ki-67 and anti-BrdUrd in a series of human malignancies. A wide range of Ki-67 and BrdUrd labelling was found, the latter generally exceeding the former. The ratio of BrdUrd to Ki-67 positive cells had a mean of 0.58 and the two parameters had a high linear correlation coefficient ($r=0.89$). This same approach was used by Kawauchi *et al.* (1989) on NHL with nearly identical results for labelling ratio and a high correlation between the two methods.

Research Objectives

Objectives Established for this Research

1. Development of a method for simultaneous staining of Ki-67 and DNA
2. Evaluation of the expression of Ki-67 in non-Hodgkin's lymphoma
3. Correlation of Ki-67 expression with DNA analysis in human solid tumors

Development of a Method for Simultaneous Staining of Ki-67 and DNA

Since the production of Ki-67 in 1983, over 150 studies have been published examining the expression of Ki-67 by immunocytology techniques, but only 12 studies have used flow cytometry to measure Ki-67. In a comparison of these 12 studies, five different fixation techniques were used to permeabilize the cells examined. Also, the commercial producer of this antibody states alcohols destroy the antigen and four of the studies were performed using alcohols. Thus, there appears to be no consensus as to the most effective fixation technique. Also noted was the use of unstained cells, or cells stained only with secondary antibody in indirect procedures, as the negative control in 11 of these studies. The only study using an irrelevant, isotypic, mouse monoclonal antibody as a negative control was one in which an alcohol was used as the fixative. For these reasons, it is imperative to develop the most effective preparatory and fixation technique to use for simultaneous quantitation of Ki-67 and DNA content by flow cytometry.

Evaluation of the Expression of Ki-67 in Non-Hodgkin's Lymphoma

All studies of Ki-67 expression measured by flow cytometry, but one, were performed measuring Ki-67 expression in cell lines or stimulated lymphocytes. This results in the expected values being at the extremes of 100% or 0% Ki-67 expression, with no values expected between these extremes. The one study examining NHL used periodate-lysine-paraformaldehyde, a fixation technique seldom cited in the literature for use in flow cytometry. For these reasons, it is

necessary to evaluate Ki-67 expression in NHL as measured by flow cytometry, utilizing the fixation technique developed, and to determine the correlation of these results with the traditional microscope-based immunocytology technique.

Correlation of Ki-67 Expression with DNA Analysis in Human Solid Tumors

S-phase analysis by measuring DNA content using flow cytometry has become established as the primary method for quantitation of proliferating cells. The few limitations associated with measurement of the S-phase could be eliminated by the performance of multiparameter flow cytometry using DNA staining in combination with a marker for cellular proliferation. Since Ki-67 has emerged in the literature in recent years as a useful marker for detection of proliferating cells, it follows that examination of the correlation between Ki-67 expression and DNA analysis both measured by flow cytometry in NHL and other solid tumors should be carried out.

MATERIALS AND METHODS

Specimens Analyzed

Fresh tissues obtained from the lymph node biopsies of 30 patients diagnosed as having non-Hodgkin's lymphoma, nine biopsies identified as breast tumors, and eight biopsies identified as colon tumors were included in this study.

Cell Lines

K562 Cell Line

The human erythroleukemia cell line K562 (ATCC, Rockville, MD) was grown in RPMI-1640 (GIBCO, Grand Island, NY) containing 15% heat-inactivated, defined fetal calf serum (Hyclone Laboratories, Logan, UT) supplemented with L-glutamine (2 mM), HEPES (10 mM), penicillin G (100 units/ml), streptomycin sulfate (100 μg/ml), and gentamicin sulfate (500 μg/ml), all from GIBCO (Grand Island, NY). The cells were cultured at 37°C with a 7% CO_2 atmosphere. Cells were maintained in log phase growth by subculturing two to three times weekly with passage at a concentration of 1 X 10^5 cells/ml. All experiments were performed with cells in log phase growth.

NALM Cell Line

The human T-cell acute leukemia cell line NALM was an original gift from T. Minowada and was cultured the same as the K562 cell line.

Epstein-Barr Virus Transformed Lymphocytes

Fourteen ml of heparinized (Becton Dickinson, Rutherford, NJ) peripheral blood were processed as per the procedure for peripheral blood lymphocytes (see below). T-cells were removed from the resulting cell suspension by sheep red blood cell (Baxter Scientific, Ocala, FL) rosetting prior to Epstein-Barr Virus (EBV) transformation. Sheep red blood cells were treated with 0.14M 2-amionethylisothiouronium bromide hydrobromide (Sigma, St Louis, MO), pH 8.0, for 30 min at 37 C prior to rosetting. After removal of T-cells, the B-cells remaining were cultured with EBV at a density of 2 X 10^6 cells/ml in RPMI-1640 medium containing 10% FCS, 5% heat-inactivated, defined equine serum (Hyclone Laboratories, Logan, UT), HEPES (10 mM), L-glutamine (2 mM), penicillin G (100 units/ml), streptomycin sulfate (100 μg/ml), gentamicin sulfate (500 μg/ml), and 2-mercaptoethanol (55 μM)(GIBCO, Grand Island, NY). EBV from the B95-8 marmoset cell line (ATCC, Rockville, MD) was obtained by harvesting tissue culture supernatant from B95-8 cells grown to confluence. Supernatant containing EBV was added at a volume of 200 μl per ml of cell suspension. The cells were cultured at 37°C with a 7% CO_2 atmosphere. Cells were fed by replacing one-half of the spent medium with fresh culture medium every 4 to 5 days until the cultures began to transform.

Once transformation had occurred, cells were maintained in log phase growth by subculturing twice weekly with passage at a concentration of 1 X 10^5 cells/ml.

Specimen Preparation

Peripheral Blood Lymphocytes

Seven ml of peripheral blood were drawn into a vacutainer tube containing EDTA (Becton Dickinson, Rutherford, NJ). Blood was transferred to a 15 ml conical tube and diluted with 3.5 ml RPMI-1640. Four ml of Lymphocyte Separation Media (LSM) (Organon Teknika, Durham, NC) was layered on the bottom of the tube by adding it through a pipet with the tip resting on the bottom. The tube was centrifuged for 20 min at 700g. The media on top was aspirated to within 1 cm of the media/LSM interface. Cells at the interface were collected with a pipet and transferred to a new 15 ml conical tube. Five ml of phosphate buffered saline (PBS) was added and a cell count performed. The tube was centrifuged for 5 min at 500g and cell pellet resuspended in sufficient RPMI-1640 with 10% newborn calf serum (NCS) (Sigma, St Louis, MO) to result in a concentration of 1 X 10^6 cells/ml.

Phytohemagglutinin Stimulated Lymphocytes

Fourteen ml of heparinized (Becton Dickinson, Rutherford, NJ) peripheral blood were processed per procedure for PBL except cells were counted and suspended at 1 X 10^6 cells/ml in RPMI-1640 plus 10% NCS and 50 mg/ml gentamicin. Half of the resulting suspension was transferred into each of

two T-25 flasks. One vial of phytohemagglutinin-M (GIBCO Grand Island, NY) was reconstituted with 5 ml sterile water. Two hundred fifty µl of phytohemagglutinin-M (PHA-M) was added to one flask and 100 µl was added to the other flask. Flasks were incubated for 72 hours at 37°C with a 7% CO_2 atmosphere. Viability of each flask was determined by trypan blue exclusion (GIBCO, Grand Island, NY) and the flask with the highest viability was utilized in experiments.

Lymphoma and Tumor Specimens

Grossly hemorrhagic, fibrotic, or necrotic tissue was trimmed from specimens. The bottom of a 60 x 15 mm plastic petri dish was coated with 1.0 ml NCS and then 5 ml RPMI-1640 was added and mixed with the NCS. Tissue was added to the petri dish. While holding the tissue with toothed forceps, it was minced with scissors. Using a transfer pipet, tissue fragments were aspirated and added to a holder having a screen on the bottom. Tissue was gently rolled with a pestle against the screen and holder sitting in the petri dish. Cells released into the medium were transferred to a 50 ml conical tube. If the cell concentration appeared dense, cells were diluted with RPMI-1640. An aliquot of cells was assayed for viability by trypan blue exclusion and a cell count performed. Cells were centrifuged (10 min at 500g), supernatant aspirated, and cell button resuspended to a concentration of 1 X 10^6 cells/ml in RPMI-1640 plus 10% NCS.

Cytospins

For each specimen examined by immunocytology, 100 µl of a cell suspension at a concentration of 1.1 X 10^6 cells/ml was placed in a cytospin cup. 100 µl of NCS was then added to each cup. Cups and slides were then placed in a Shandon Cytospin centrifuge (Shandon Southern Products, Pittsburgh, PA) were they were spun for 5 sec once 16,000 rpm was reached. Slides were removed from the centrifuge, allowed to air dry for 10 min, were fixed with acetone (Fisher, Fair Lawn, NJ) for 5 min, and allowed to air dry for 10 min. Slides were immediately stored at -70°C until stained.

Monoclonal Antibody Ki-67

The anti-Ki-67 monoclonal mouse antibodies utilized in this research are commercially available from Dakopatts, Carpinteria, CA. These antibodies are both of the IgG1, kappa subclass and are obtained from tissue culture supernatant dialyzed against 0.05M Tris/HCl, pH 7.2, with 15 mM NaN_3. The FITC-conjugated antibody had a mouse IgG concentration of 100 µg/ml as determined by single radial immunodiffusion. The FITC/protein ratio of the conjugated antibody is approximately 6:1 as determined by spectrophotometry. 100 µl of antibody was utilized after diluting 1:10 in 20% NCS in PBS for a final concentration of 1 µg per test. The unconjugated antibody had a mouse IgG concentration of 292 µg/ml as determined by single radial immunodiffusion. 100 µl of antibody was utilized after diluting 1:10 or 1:33 in 20% NCS in PBS for a final concentration of 3 µg per test or 1 µg per test respectively.

Fixation

Acetone Fixation

For each specimen tested, 1 ml of cells in suspension at 1 X 10^6 cells/ml was pelleted (5 min at 500*g*) in each of two tubes. The cells were fixed in 1 ml 95% acetone in PBS for 10 min at room temperature. The cells were pelleted and washed in 1 ml of PBS (5 min at 500*g* and 4°C). One tube of cells was incubated with 100 µl FITC-conjugated monoclonal antibody (MoAb) Ki-67 (1:10 in PBS with 20% NCS) for 15 min at 4°C. The other tube of cells was incubated with 100 µl FITC-conjugated irrelevant, isotypic, mouse monoclonal antibody (mIgG)(1:5 in PBS with 20% NCS) (Becton Dickinson, San Jose, CA) for 15 min at 4°C. The cells were pelleted and washed in 1 ml of PBS (5 min at 500*g* and 4°C). Cells were resuspended in 250 µl RNase (8.33 Kunitz units/ml in 1.12% sodium citrate in dH_2O) (Sigma, St Louis, MO) and incubated for 30 min at 37°C. Two hundred fifty µl of isotonic propidium iodide (PI) (50 µg/ml in 1.12% sodium citrate in dH_2O) (Calbiochem, La Jolla, CA) was added to the RNase and incubated for 30 min at room temperature. Cells were filtered through 44 µm mesh and analyzed within 60 min by flow cytometry.

Periodate-Lysine-Paraformaldehyde Fixation

For each specimen tested, 1 ml of cells in suspension at 1 X 10^6 cells/ml was pelleted (5 min at 500*g*) in each of two tubes. The cells were fixed in 1 ml periodate-lysine-paraformaldehyde (PLP) for 15 min at -10°C. PLP fixative

consisted of 37mM phosphate buffer saline at pH 7.4 containing 75mM sodium m-periodate, 10mM lysine monohydrochloride (both from Sigma, St Louis, MO), and 2% paraformaldehyde (Eastman Kodak, Rochester, NY). The cells were pelleted and washed in 1 ml of PBS (5 min at 500*g* and 4°C). One tube of cells was incubated with 100 μl FITC-conjugated MoAb Ki-67 (1:10 in PBS with 20% NCS) for 15 min at 4°C. The other tube of cells was incubated with 100 μl FITC-conjugated irrelevant monoclonal mIgG antibody (1:5 in PBS with 20% NCS) for 15 min at 4°C. The cells were pelleted and washed in 1 ml of PBS (5 min at 500*g* and 4°C). Cells were resuspended in 250 μl RNase (8.33 Kunitz units/ml in 1.12% sodium citrate in dH_2O) and incubated for 30 min at 37°C. 250 μl of isotonic PI (50 μg/ml in 1.12% sodium citrate in dH_2O) was added to the RNase and incubated for 30 min at room temperature. Cells were filtered through 44 μm mesh and analyzed within 60 min by flow cytometry.

Paraformaldehyde/Triton X-100/Ethanol Fixation

For each specimen tested, 1 ml of cells in suspension at 1 X 10^6 cells/ml was pelleted (5 min at 500*g*) in each of two tubes. The cells were fixed in 1 ml 0.5% paraformaldehyde in PBS for 5 min at 4°C. Cells were soaked in 1 ml 0.1% Triton X-100 in PBS (Sigma, St Louis, MO) for 10 min at 4°C and pelleted (5 min at 500*g*). One tube of cells was incubated with 100 μl FITC-conjugated MoAb Ki-67 (1:10 in PBS with 20% NCS) for 15 min at 4°C. The other tube of cells was incubated with 100 μl FITC-conjugated irrelevant monoclonal mIgG antibody (1:5 in PBS with 20% NCS) for 15 min at 4°C. The

cells were pelleted and washed in 1 ml of PBS (5 min at 500*g* and 4°C). Cells were fixed in 70% ethanol in PBS (AAPER, Shelbyville, KY) for 10 min at 4°C and pelleted (5 min at 500*g*). Cells were soaked in 0.02% Triton X-100 in 1.12% sodium citrate (in dH_2O) for 20 min at 4°C and pelleted (5 min at 500*g*). The cells were pelleted and washed in 1 ml of PBS (5 min at 500*g* and 4°C). Cells were resuspended in 250 µl RNase (8.33 Kunitz units/ml in 1.12% sodium citrate in dH_2O) and incubated for 30 min at 37°C. 250 µl of isotonic PI (50 µg/ml in 1.12% sodium citrate in dH_2O) was added to the RNase and incubated for 30 min at room temperature. Cells were filtered through 44 µm mesh and analyzed within 60 min by flow cytometry.

Ethanol Fixation

For each specimen tested, 1 ml of cells in suspension at 1 X 10^6 cells/ml was pelleted (5 min at 500*g*) in each of two tubes. The cells were fixed in 1 ml 50% ethanol in RPMI-1640 for 15 min at 4°C. The cells were pelleted (5 min at 500*g*), soaked in 1 ml of PBS for 15 min at 4°C, and pelleted (5 min at 500*g*) again. One tube of cells was incubated with 100 µl FITC-conjugated MoAb Ki-67 (1:10 in PBS with 20% NCS) for 15 min at 4°C. The other tube of cells was incubated with 100 µl FITC-conjugated irrelevant monoclonal mIgG antibody (1:5 in PBS with 20% NCS) for 15 min at 4°C. The cells were pelleted and washed in 1 ml of PBS (5 min at 500*g* and 4°C). Cells were resuspended in 250 µl RNase (8.33 Kunitz units/ml in 1.12% sodium citrate in dH_2O) and incubated for 30 min at 37°C. 250 µl of isotonic PI (50 µg/ml in 1.12% sodium citrate in

dH_2O) was added to the RNase and incubated for 30 min at room temperature. Cells were filtered through 44 μm mesh and analyzed within 60 min by flow cytometry.

1.0% Paraformaldehyde/Triton X-100

For each specimen tested, 1 ml of cells in suspension at 1 X 10^6 cells/ml was pelleted (5 min at 500*g*) in each of two tubes. The cells were fixed in 1 ml 1.0% paraformaldehyde in PBS for 15 min at 4°C. Cells were soaked in 1 ml 0.1% Triton X-100 in PBS for 10 min at 4°C and pelleted (5 min at 500*g*). The cells were washed in 1 ml of PBS (5 min at 500*g* and 4°C). One tube of cells was incubated with 100 μl FITC-conjugated MoAb Ki-67 (1:10 in PBS with 20% NCS) for 15 min at 4°C. The other tube of cells was incubated with 100 μl FITC-conjugated irrelevant monoclonal mIgG antibody (1:5 in PBS with 20% NCS) for 15 min at 4°C. The cells were pelleted and washed in 1 ml of PBS (5 min at 500*g* and 4°C). Cells were resuspended in 250 μl RNase (8.33 Kunitz units/ml in 1.12% sodium citrate in dH_2O) and incubated for 30 min at 37°C. 250 μl of isotonic PI (50 μg/ml in 1.12% sodium citrate in dH_2O) was added to the RNase and incubated for 30 min at room temperature. Cells were filtered through 44 μm mesh and analyzed within 60 min by flow cytometry.

0.5% Paraformaldehyde/Triton X-100

For each specimen tested, 1 ml of cells in suspension at 1 X 10^6 cells/ml when no cytospin was prepared, or 1.1 X 10^6 cells/ml when a cytospin was

prepared, was pelleted (5 min at 500g) in each of two tubes. The cells were fixed in 1 ml 0.5% paraformaldehyde in PBS for 10 min at 4°C. Cells were soaked in 1 ml 0.1% Triton X-100 in PBS for 3 min at 4°C and pelleted (5 min at 500g). On specimens for immunocytology, 100 μl of cell suspension was removed during this soak for cytospin preparation and 100 μl of 0.1% Triton X-100 was added to the flow cytometry sample. One tube of cells was incubated with 100 μl FITC-conjugated MoAb Ki-67 (1:10 in PBS with 20% NCS) for direct testing, or 100 μl unconjugated MoAb Ki-67 (1:33 in PBS with 20% NCS) for indirect testing, for 15 min at 4°C. The other tube of cells was incubated with 100 μl FITC-conjugated irrelevant monoclonal mIgG antibody (1:5 in PBS with 20% NCS) for direct testing, or 100 μl unconjugated irrelevant monoclonal mIgG antibody (1:100 in PBS with 20% NCS) (Coulter, Hialeah, FL) for indirect testing, for 15 min at 4°C. For indirect testing, cells were pelleted and washed in 1 ml of PBS (5 min at 500g and 4°C) and then incubated with 100 μl of sheep anti-mouse FITC-conjugated antibody (1:500 in NCS) (Cappel, Malvern, PA) for 15 min at 4°C. Whether direct or indirect testing, cells were now pelleted and washed in 1 ml of PBS (5 min at 500g and 4°C). Cells were resuspended in 250 μl RPMI-1640 and 250 μl of isotonic PI (50 μg/ml in 1.12% sodium citrate in dH_2O) was added with incubation for 30 min at room temperature. Cells were filtered through 44 μm mesh and analyzed within 60 min by flow cytometry.

Immunocytology

Cytospin slides were removed from storage at -70°C and allowed to warm at room temperature for 30 min. Slides were placed in PBS for 5 min, cytospin spots were covered with normal horse serum (1:100 in 2% bovine serum albumin (BSA) in PBS), and slides were incubated 2 min. Slides were tilted to remove excess normal horse serum. Cytospin spots were covered with MoAb Ki-67 (1:200 in 20% NCS in PBS) and incubated at room temperature for 15 min. Slides were rinsed with PBS. Cytospin spots were covered with biotinylated horse anti-mouse (1:125 in PBS) (Vector Laboratories, Burlingame, CA) and incubated at room temperature for 10 min. Slides were rinsed with PBS. Cytospin spots were covered with Avidin DH:Biotinylated Horseradish Peroxidase mixture (each reagent 1:125 in PBS) (Vector Laboratories, Burlingame, CA) and incubated at room temperature for 10 min. Slides were rinsed with PBS. Peroxidase substrate solution was prepared by dissolving 10 mg diaminobenzidene (DAB) in 10 ml of 50 mM Tris-HCl buffer at pH 7.6 and then filtering the solution. 8 drops of a 3% H_2O_2 solution (100 µl 30% H_2O_2 in 10 ml dH_2O) was then added to the DAB solution (all reagents Sigma, St Louis, MO). Cytospin spots were covered with DAB solution and incubated at room temperature for 5 min. Slides were rinsed with running water for 10 min, counterstained with hematoxylin for 20 sec, and washed 5 times with dH_2O. Slides were then dehydrated through graded alcohols, cleared with xylene, and coverslipped with permanent media. A positive control slide of the K562 cell line and a negative control slide of PBL were included each time slides were

stained. Ki-67 was quantified by determining the percentage of positive cells (brown colored cells) among the total number of cells (negative blue colored plus positive brown colored cells) using an oil immersion objective. Field selection sought areas of strongest Ki-67 staining intensity evident by lower power scanning. The total cell count performed on each slide was 500 cells.

Flow Cytometry

Samples were measured on a FACStar Plus or FACStar flow cytometer (Becton Dickinson, Mountain View, CA) equipped with an argon-ion laser emitting at a wavelength of 488 nm. FITC fluorescence was measured using a 530/30 nm bandpass filter and PI fluorescence was measured using a 570/40 nm bandpass filter. Alignment of the flow cytometer was performed using PI stained chicken erythrocyte nuclei (CEN) prepared in this lab. The same voltage and gain settings, initially determined using PBL, were utilized each day for forward and side light scatter measurements. The instrument was standardized each day for mean green fluorescence with 1.5 μM green fluorescent microspheres (Polysciences, Warrington, PA) and for mean red fluorescence with PI stained CEN. Red fluorescence was used as the trigger, with a threshold setting of 200, FL1-FL2 compensation was set to 0.6, and a laser power setting of 0.250 watts used. 20,000 events were counted for each sample and the results were stored in list mode files.

Data Analysis

List mode data were analyzed using Consort 30, LYSYS, or FACStar Plus Research software (Becton Dickinson, Mountain View, CA). Initially no gates were used to exclude cells. In later experiments when pulse processing was utilized, initial gating consisted of exclusion of cells which were not singlets on a dot plot of red fluorescence area on the X axis (linear scale) and red fluorescence width on the Y axis (linear scale). Two-parameter correlated data were displayed as dot plots, with DNA content shown as height of red fluorescence on the X axis (linear scale) and Ki-67 expression shown as height of green fluorescence on the Y axis (log scale). Gated dot plots of mIgG controls and Ki-67 stained cells were compared to determine values to use as markers for quantitation of Ki-67 on subsequent FITC histograms. A single-parameter FITC histogram of the gated cell population was displayed to set markers to quantitate Ki-67 positive and Ki-67 negative cell populations. A single-parameter DNA histogram of the gated cell population was displayed to set markers to allow the quantitation of G0/G1, S, and G2/M phases of the cell cycle.

Statistical Analysis

Correlations between resulting data were calculated using linear regression. Regression analysis results and scatter plots of the best fit line were produced using the data regression function of Lotus 1-2-3 (Lotus Development Corp., Cambridge, MA).

RESULTS

Determination of Fixation Technique

Studies in the literature were utilized to determine the fixation techniques to initially examine. No consensus existed as to the most effective fixation technique from the 12 studies examining Ki-67 expression by flow cytometry. However, eight of the these studies used one of three techniques: acetone, periodate-lysine-paraformaldehyde (PLP), or paraformaldehyde/triton/ethanol (PTE). Each technique was evaluated for effectiveness by examining the percentage of Ki-67 expression, quality of DNA staining, and ratio of the mean fluorescence of Ki-67 positive and negative populations compared to the control mean fluorescence of cells exposed to an irrelevant, isotypic, mouse monoclonal antibody (mIgG). Representative histograms resulting from flow cytometric analysis of a specimen and the use of the histograms to calculate the factors evaluated are shown in Figure 1.

The cell line samples tested were estimated to have 100% of the cells expressing Ki-67 since a cell line should consist almost exclusively of proliferating cells. The normal blood lymphocytes tested were estimated to have 0% of the cells expressing Ki-67 since these are not proliferating cells. The cell mixtures tested were estimated to have 50% of the cells expressing Ki-67 since these

consisted of a suspension of 50% normal lymphocytes and 50% cells from a cell line.

Staining for DNA content was performed simultaneously with staining for Ki-67 expression on the different cell types. DNA histograms with low coefficients of variation (CVs) for the G0/G1 peak were established as a requirement, since one of the final objective of this research was to examine the relationship between DNA content and Ki-67 expression. For this research, a DNA G0/G1 peak CV of 3 ± 1% was the desired goal. The DNA staining also served as an indication of the effectiveness of cell permeabilization because of the relative ease with which entry of PI into the nucleus can be achieved compared to entry of an antibody.

Assuming that Ki-67 is exclusively expressed in cycling cells, an effective fixation technique should result in the mean fluorescence for a Ki-67 negative population (e.g. nonproliferating cells) being similar to the mean fluorescence for the mIgG negative control, and a Ki-67 positive population (e.g. highly proliferative cells) having as great a change in mean fluorescence value from the control as possible.

The results of analysis using these three techniques on cell lines, normal lymphocytes, and mixtures of both cell types are summarized in Table 1. Acetone and PLP fixation resulted in mean percentages of Ki-67 positive cells far from the expected results for both cell lines and cell mixtures. Normal lymphocytes had little Ki-67 expression detected by either, but due to the poor positive results, little confidence can be placed in these values. The mean DNA

G0/G1 peak CVs obtained with acetone were not in the range desired and those of PLP were of an unacceptable level.

PTE was the most effective of these three fixatives because it detected the highest expression of Ki-67 in cell lines, had the lowest detection of Ki-67 expression in normal lymphocytes, had the largest separation between positive and negative Ki-67 populations, and had DNA G0/G1 peak CVs closest to those desired.

Tests using PTE fixative at differing concentrations of paraformaldehyde and without ethanol were conducted since paraformaldehyde and Triton X-100 treatment of cells can produce satisfactory DNA G0/G1 peak CVs and the commercial producer of the Ki-67 antibody states alcohols can destroy the Ki-67 antigen. Thus, ethanol in the PTE fixative could be contributing to the higher DNA G0/G1 peak CVs and lower percentage of positive cells than expected. However, four of the 12 published studies used alcohol fixation to permeabilize cells for detection of Ki-67 by flow cytometry, because poor results were obtained with acetone fixation. Ethanol fixation was evaluated to determine its effectiveness in detection of Ki-67, because of these conflicting reports. The results of analysis are summarized in Table 2. Ethanol and 1.0% PT fixation resulted in much better detection of Ki-67 expressing cells for the cell lines and cell mixtures than the acetone, PLP, or PTE fixatives. Little detection of Ki-67 expression by either occurred in the normal lymphocytes. The mean CVs for DNA were not as good as desired for ethanol alone and those of 1.0% PT were sufficiently large to be considered unacceptable.

Fixation with 0.5% PT was the most effective technique for detection of Ki-67 expression and DNA staining of the fixatives evaluated. This fixative resulted in one of the two highest means of fluorescence for Ki-67 expressing cells. In addition, it was the most accurate in determining the correct percentage of Ki-67 expressing cells in the cell mixtures, produced the lowest detection of Ki-67 expression in normal lymphocytes, and the DNA G0/G1 peak CVs were the lowest observed. In all criteria used for evaluation of fixation, this technique had the smallest standard deviations associated with the measurements. For these reasons, fixation with 0.5% PT was the technique used in the analysis of human tumor samples.

Nonspecific Protein Staining

An increase in the fluorescent intensity was seen from the mean fluorescence for the mIgG negative control to the mean fluorescence of the negative population of the Ki-67 antibody stained samples in all the fixatives. One possible explanation for this increase is nonspecific staining of cellular protein by unconjugated FITC in the MoAb Ki-67 reagent. To evaluate this possibility, a sample of spleen from a patient with hairy-cell leukemia was analyzed. These cells have very abundant cytoplasm and a low proliferation rate. The staining of the hairy-cell lymphocytes was compared to normal lymphocytes and PHA-stimulated lymphocytes. The ratios resulting are listed in Table 3.

The ratios for the hairy-cell leukemia were lower than both normal lymphocytes and PHA-stimulated lymphocytes. Higher ratios for the hairy-cell leukemia would have been expected to result had nonspecific protein staining by FITC occurred. Therefore, it was concluded the difference observed is not a result of nonspecific staining by unconjugated FITC to cellular protein.

Direct Versus Indirect Procedures

A second possible explanation for the increase in the fluorescence of the negative population is nonspecific binding of the Ki-67 antibody. This could result from differences in the FITC/protein ratio of the FITC-labelled mIgG negative control and FITC-labelled Ki-67 antibody reagents. To evaluate this possibility, direct and indirect staining procedures were compared. Indirect staining can detect nonspecific binding, because the FITC/protein ratio will be identical for the control and Ki-67 samples since the same FITC-conjugated secondary antibody is used. Results of this comparison are listed in Table 4 and indicate indirect staining leads to improvement in the differences. For this reason, indirect staining was utilized to analyze the human tumor specimens.

Antibody Titration

A third possible explanation for the increase in the negative population fluorescence is variation in the methods used for quantitation of the antibody in the primary antibody reagents. This could result in differences in the amount of control and Ki-67 antibodies used per test. To evaluate this possibility titration

of all antibodies utilized was performed to determine the optimum antibody concentrations to use. The titration results are listed in Table 5.

The titration results for the mIgG and Ki-67 antibodies were essentially equal in the K562 cell line with the sheep anti-mouse secondary antibody dilution 1:500 producing the most difference in the positive cells and the least difference in the negative cells. It was concluded therefore the optimal concentrations for testing human tumors was with the primary antibodies at a concentration of 1μg/test and the secondary antibody at a dilution of 1:500. Use of the antibodies at these concentrations failed to further decrease the change in mean fluorescence observed in the negative cell populations.

Specimen Storage Conditions

The storage conditions utilized for the human tumor samples were investigated to determine if they had contributed to the increase in the negative population fluorescence seen. To evaluate these conditions, refrigeration over time and the serum concentration of the storage media were monitored with a human tonsil specimen. The results of this study are listed in Table 6.

Changes in the Ki-67 negative and positive populations were essentially the same regardless of the length of refrigeration or serum concentration. An increased ratio of the Ki-67 negative population fluorescence to the mIgG negative control occurred for 15% NCS. Since 10% NCS was used on human tumor specimens and the results for this fixative on cell mixtures was nearly identical to the 5% serum concentration results, it was concluded that

refrigeration and serum concentration did not contribute to the difference observed between the mIgG negative control and the Ki-67 negative populations.

Ki-67 Flow Cytometry Correlation with Immunocytology

Using the fixation technique developed, the percentages of Ki-67 expression in 12 lymph node biopsies from patients with NHL were determined. Representative dot plots from flow cytometric analysis of these specimens, with FL1 being Ki-67 expression and FL2 being DNA staining, are shown in Figure 2.

Cytospin slides prepared from the same cell suspensions analyzed by flow cytometry were stained by an immunocytology procedure using the same Ki-67 antibody reagent and the percentages of Ki-67 expression were determined. The data obtained were compared by linear regression and the correlation between the two data sets was calculated. The correlation resulting is shown in Figure 3.

Excellent correlation between the percentages of Ki-67 expression obtained by immunocytology and those by flow cytometry resulted ($r=0.91$). It was concluded the fixation technique developed was satisfactory for the determination of Ki-67 expression in NHL using flow cytometry.

Ki-67 Expression in Non-Hodgkin's Lymphoma

The percentage of Ki-67 expression and the DNA S-phase were determined by simultaneous staining and flow cytometric analysis for 30 lymph node biopsies obtained from patients with NHL. The data obtained were

compared by linear regression and the correlation between the two data sets was calculated. The correlation resulting is shown in Figure 4.

Excellent correlation between the percentage of Ki-67 expression and DNA S-phase resulted (r=0.95). It was concluded the simultaneous staining for Ki-67 expression and DNA S-phase was satisfactory and a linear relationship between the proliferation parameters existed in NHL.

Ki-67 Expression in Breast and Colon Tumors

The expression of Ki-67 and the DNA S-phase were examined in solid tumors other than lymphomas. Biopsy specimens from nine breast tumors and eight colon tumors were analyzed using the fixation technique developed. The Ki-67 expression and DNA S-phase measured for each tumor type were compared by linear regression and the correlation between the two data sets for each type tumor was calculated. The correlation resulting for the breast tumors is shown in Figure 5 and the correlation for colon tumors in Figure 6.

No significant correlation was found between Ki-67 expression and DNA S-phase in either the breast tumors (r=0.19) or the colon tumors (r=0.53). The lack of correlation may be due to further development in fixation technique being needed before simultaneous staining for Ki-67 expression and DNA S-phase can be applied to solid tumors other than lymphoma.

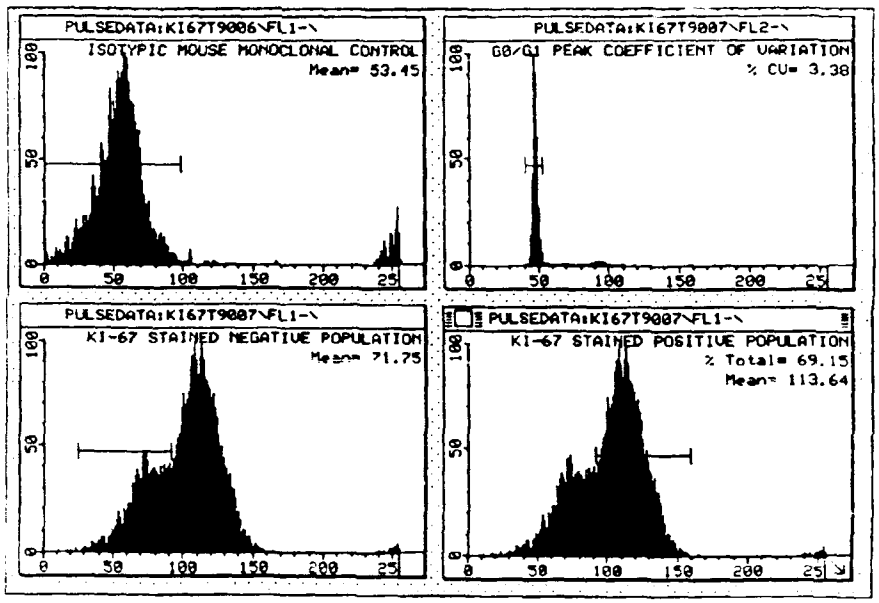


Figure 1. Representative Histograms of Cells Exposed to either FITC-Labelled Mouse Monoclonal IgG (Negative Control) or Ki-67, and Stained Simultaneously for DNA (PI). For each histogram, fluorescent intensity is on the x-axis and relative cell number is on the y-axis. FL1 indicates green (FITC) fluorescence and FL2 indicates red (PI) fluorescence. The percentage of positive cells evaluated was calculated by dividing the number of Ki-67 stained cells with a fluorescent intensity greater than the maximum fluorescent intensity of the isotypic mouse monoclonal control by the total number of cells in the histogram and multiplying by 100. The DNA coefficient of variation (CV) evaluated was determined from the G0/G1 peak of the red (PI) fluorescence histogram. The negative ratio evaluated was calculated by dividing the mean fluorescent intensity of the Ki-67 stained negative population by the mean fluorescent intensity of the isotypic mouse monoclonal control. The positive ratio evaluated was calculated by dividing the mean fluorescent intensity of the Ki-67 stained positive population by the mean fluorescent intensity of the isotypic mouse monoclonal control.

Table 1. Fixation Results for Acetone, PLP, and PTE.

		Acetone	PLP[a]	PTE[b]
Cell Lines	Percent Positive	10.00[c] ± 1.63 (3)	19.78 ± 13.62 (9)	78.17 ± 19.78 (6)
	Negative Ratio[d]	1.06 ± 0.08 (3)	1.18 ± 0.33 (9)	4.89 ± 4.10 (6)
	Positive Ratio[e]	1.54 ± 0.23 (3)	1.90 ± 1.63 (9)	11.00 ± 12.23 (6)
	DNA C.V.[f]	6.00 ± 0.58 (6)	11.00 ± 2.67 (16)	5.17 ± 1.16 (12)
Normal Lymphocytes	Percent Positive	1.00 ± 0.00 (3)	2.33 ± 1.89 (6)	2.50 ± 1.66 (4)
	Negative Ratio	1.00 ± 0.04 (3)	1.36 ± 0.57 (6)	2.19 ± 0.95 (4)
	DNA C.V.	5.75 ± 0.42 (6)	10.73 ± 3.80 (9)	4.29 ± 1.26 (8)
Cell Mixtures	Percent Positive	5.00 ± 2.45 (3)	15.00 ± 10.00 (3)	Not Done
	Negative Ratio	1.32 ± 0.24 (3)	2.93 ± 0.40 (3)	Not Done
	Positive Ratio	1.59 ± 0.27 (3)	5.43 ± 1.79 (3)	Not Done
	DNA C.V.	5.46 ± 0.39 (6)	9.96 ± 1.26 (6)	Not Done

[a] Periodate-lysine-paraformaldehyde fixative
[b] Paraformaldehyde/Triton X-100/ethanol fixation
[c] Mean ± 1SD (Number performed)
[d] Ratio of Ki-67 negative fluorescence to mIgG control fluorescence
[e] Ratio of Ki-67 positive fluorescence to mIgG control fluorescence
[f] DNA coefficient of variation

Table 2. Fixation Results for Ethanol, 1.0% PT, and 0.5% PT.

		Ethanol	1.0% PT[a]	0.5% PT[b]
Cell Lines	Percent Positive	85.10[c] ± 23.07 (21)	92.82 ± 6.90 (11)	86.70 ± 10.63 (13)
	Negative Ratio[d]	1.37 ± 0.58 (20)	1.64 ± 0.70 (9)	1.35 ± 0.22 (8)
	Positive Ratio[e]	1.91 ± 0.95 (20)	2.49 ± 1.09 (11)	1.77 ± 0.41 (13)
	DNA C.V.[f]	5.05 ± 1.77 (40)	7.39 ± 2.18 (22)	4.80 ± 1.08 (26)
Normal Lymph-ocytes	Percent Positive	4.53 ± 2.04 (19)	3.40 ± 1.96 (5)	2.33 ± 1.89 (3)
	Negative Ratio	1.49 ± 0.42 (19)	1.91 ± 0.92 (5)	1.11 ± 0.08 (3)
	DNA C.V.	6.60 ± 2.29 (38)	10.39 ± 3.29 (10)	3.19 ± 0.10 (6)
Cell Mixtures	Percent Positive	54.60 ± 15.46 (5)	37.50 ± 9.63 (4)	48.50 ± 3.50 (2)
	Negative Ratio	2.15 ± 1.44 (5)	2.00 ± 0.91 (4)	1.16 ± 0.01 (2)
	Positive Ratio	2.99 ± 1.91 (5)	3.92 ± 1.53 (4)	1.87 ± 0.01 (2)
	DNA C.V.	4.37 ± 1.38 (8)	11.23 ± 2.80 (6)	3.31 ± 0.42 (4)

[a] 1.0% paraformaldehyde/0.1% Triton X-100
[b] 0.5% paraformaldehyde/0.1% Triton X-100
[c] Mean ± 1SD (Number performed)
[d] Ratio of Ki-67 negative fluorescence to mIgG control fluorescence
[e] Ratio of Ki-67 positive fluorescence to mIgG control fluorescence
[f] DNA coefficient of variation

Table 3. Non-specific Protein Staining Ratios.

Cell Type Tested	PLP[a] Fixation	Ethanol Fixation	0.5% PT[b] Fixation
Normal Lymphocytes	1.78[d]	1.87	1.48
PHA-Stimulated Lymphocytes[c]	2.40	2.09	2.06
Hairy-Cell Leukemia	1.68	1.49	1.43

[a] Periodate-lysine-paraformaldehyde fixative
[b] 0.5% paraformaldehyde/0.1% Triton X-100 fixation
[c] Phytohemagglutinin-stimulated lymphocytes
[d] Ratio of Ki-67 negative fluorescence to mIgG control fluorescence

Table 4. Direct and Indirect Staining Results.

Cell Type Tested	Direct Procedure	Indirect Procedure	Number Tested
Cell Lines	1.57[a]	1.21	6
Normal Lymphocytes	1.37	1.07	2
Cell Mixtures	1.40	1.18	2

[a] Ratio of Ki-67 negative fluorescence to mIgG control fluorescence

Table 5. Results of Antibody Titration.

		Sheep Anti-Mouse 1:50[a]	Sheep Anti-Mouse 1:100	Sheep Anti-Mouse 1:200	Sheep Anti-Mouse 1:500
K562 Cell Line	mIgG and Ki-67 at 3μg/test[b]	47.90[c]	68.06	113.97	116.80
	mIgG and Ki-67 at 1μg/test	49.03	63.22	100.36	102.72
Normal Lymph-ocytes	mIgG and Ki-67 at 1μg/test	21.51[d]	20.55	19.44	9.55

[a] Dilution of sheep anti-mouse FITC-conjugated secondary antibody in newborn calf serum (NCS)

[b] Concentration of isotypic mouse negative control or MoAb Ki-67 in 20% NCS in PBS

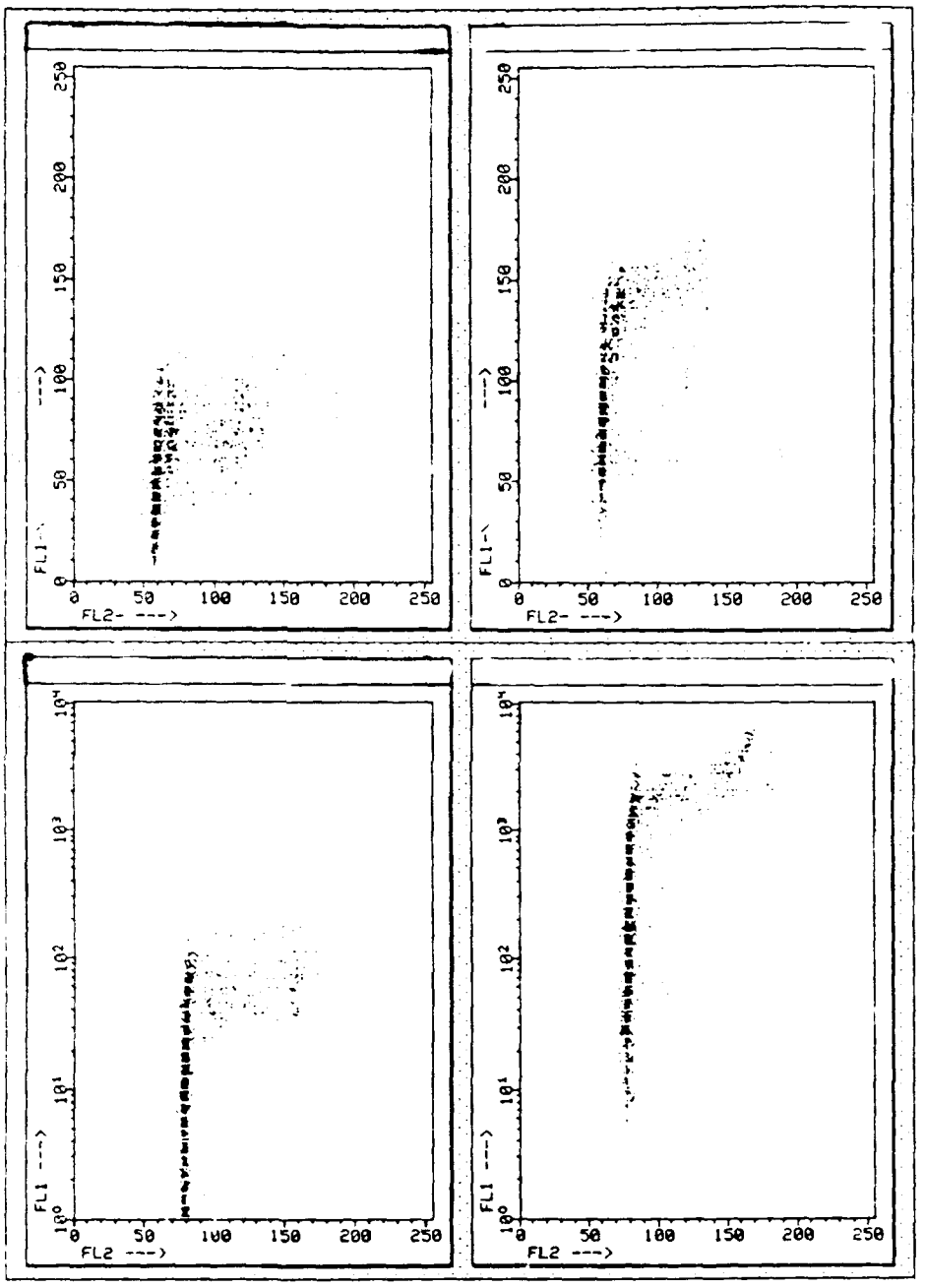
[c] Difference in mean fluorescence between mIgG negative control and Ki-67 positive population

[d] Difference in mean fluorescence between mIgG negative control and Ki-67 negative population

Table 6. Refrigeration and Serum Concentration Effects.

Hours of Refrigeration	5% NCS[a]			15% NCS		
	mIgG Cont.[b]	Ki67 Neg.[c]	Ki67 Pos.[d]	mIgG Cont.	Ki67 Neg.	Ki67 Pos.
Fresh	67	76	131	67	76	131
24 Hours	90	103	158	76	99	157
48 Hours	86	100	157	82	97	155
72 Hours	74	99	157	76	97	149

[a] Concentration of newborn calf serum in the storage media
[b] Mean fluorescence of the mIgG negative control
[c] Mean fluorescence of the Ki-67 negative population
[d] Mean fluorescence of the Ki-67 positive population



Mouse IgG Stained Samples Ki-67 Stained Samples

Figure 2. Representative Dot Plots of Ki-67 Expression in Non-Hodgkin's Lymphoma. FL1 = Ki-67 Expression / FL2 = DNA Staining.

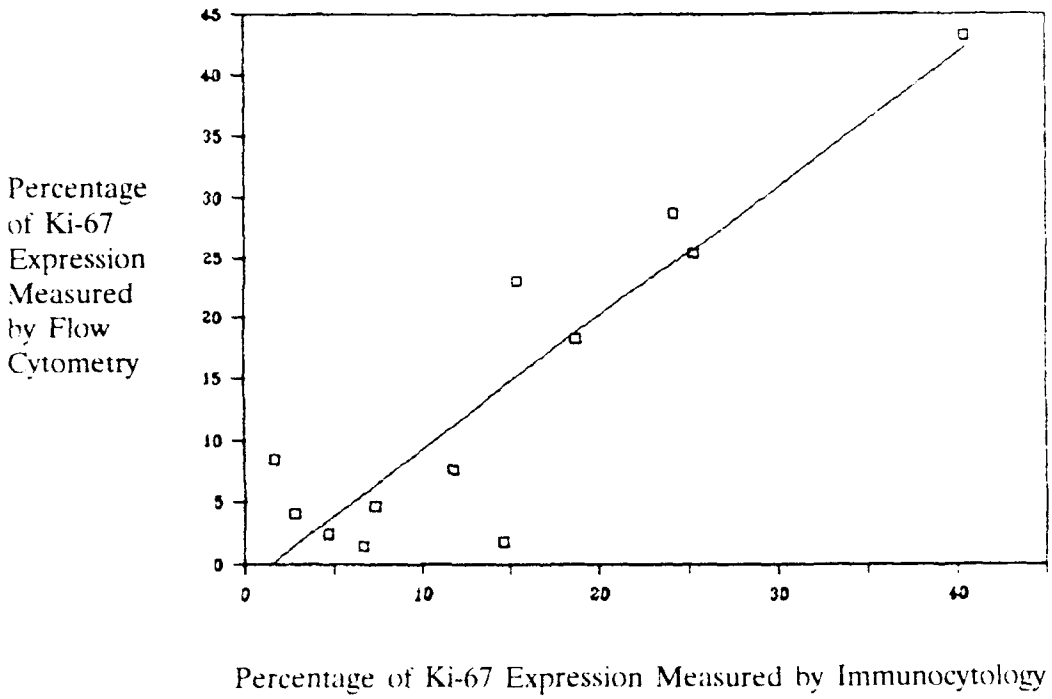


Figure 3. Correlation of Ki-67 Expression Measured by Flow Cytometry and Immunocytology. The correlation coefficient (r) = 0.91

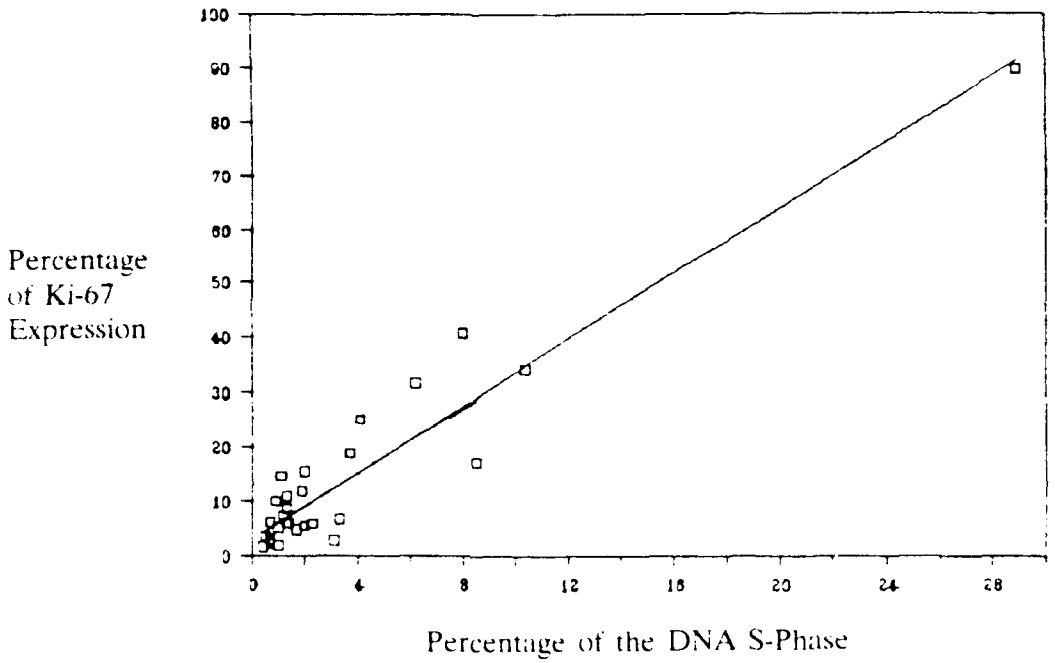


Figure 4. Correlation of Ki-67 Expression and DNA S-Phase in Non-Hodgkin's Lymphoma. The correlation coefficient (r) is 0.95

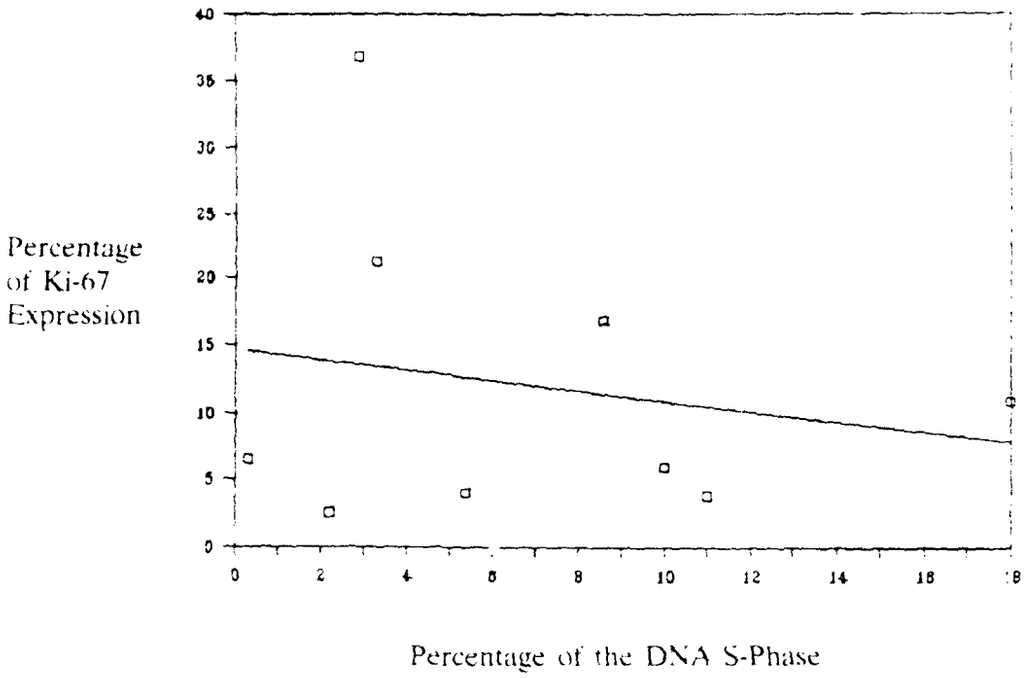


Figure 5. Correlation of Ki-67 Expression and DNA S-Phase in Breast Tumors. The correlation coefficient (r) is 0.19

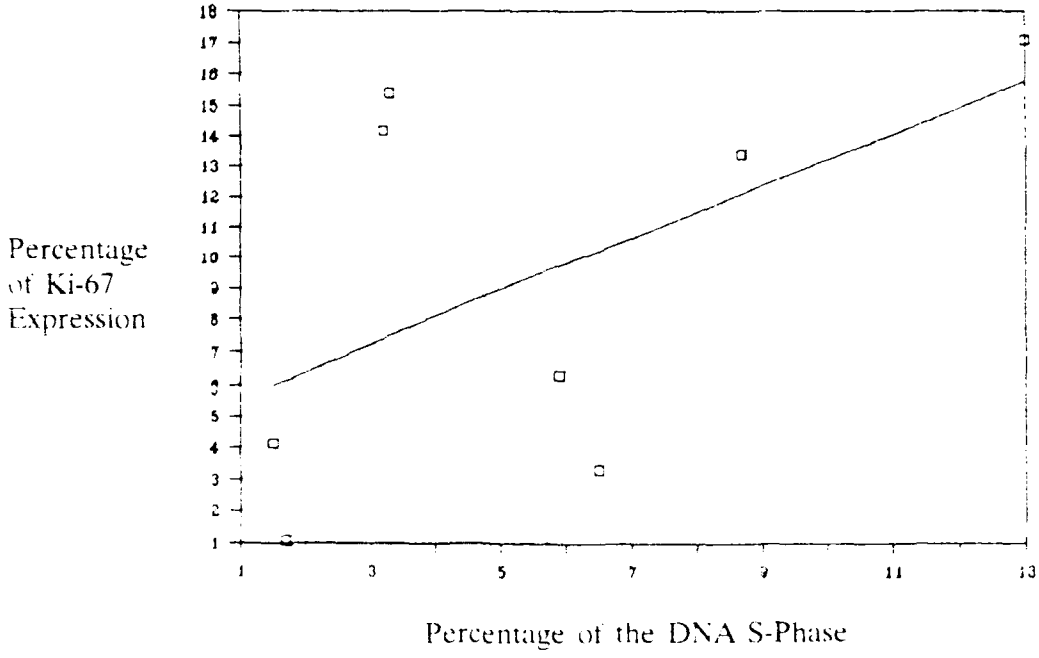


Figure 6. Correlation of Ki-67 Expression and DNA S-Phase in Colon Tumors. The correlation coefficient (r) is 0.53

DISCUSSION

Research Importance

Kinetic information resulting from the determination of cell proliferation properties is important in assessing the growth of tumors since these measurements can provide significant prognostic and therapeutic information. The measurement of DNA content by flow cytometry has become the most useful and frequently applied technique for measuring cell proliferation. From a clinical perspective a correlation of DNA S-phase with both the histological grade of tumors and the length of patient survival is observed and the S-phase fraction as a measure of cellular proliferation has been verified in comparative studies with other methods for the measurement of cellular proliferation. From a technical viewpoint DNA analysis is performed more easily than other technical techniques. For these reasons, flow cytometric analysis of DNA content is now widely employed both in research as well as in clinical laboratories.

Computer programs are normally utilized to determine the number of cells in each phase of the cell cycle from the DNA histogram. However, these programs fail when used to analyze cell suspensions having diploid and aneuploid populations not clearly separated by DNA content, tetraploid

populations without a clearly discernible S-phase and G2/M, or when multiple aneuploid populations are present. Manual interactive calculations can be performed in these cases to approximate S-phase fractions, but this can be very subjective and introduce artifactual errors in cell cycle analysis. When tumor cells are diploid or close to diploid and contain an undetermined amount of non-neoplastic cells, stromal elements, or inflammatory cells, accurate determination of tumor cell fractions using just DNA content cannot be done. However, if a separate marker unique to either the tumor or normal cell populations is available, it can be used simultaneously with DNA staining to allow differentiation of the two populations. Thus, detection of two populations with identical or nearly identical DNA content not recognized by DNA histogram analysis can be accomplished. A cellular antigen unique to cellular proliferation would be such a marker and could contribute to a more accurate estimation of cell cycle fractions.

An ideal marker of cellular proliferation would be the Ki-67 antigen. Expression of this antigen measured by immunocytology has been extensively documented in the literature. In excess of 40 studies of lymphomas and 60 studies of non-lymphoid solid tumors have been published examining the expression of Ki-67 measured by immunocytology. In these studies, close correlations with histological grade of the tumor and length of patient survival have been found to exist. In addition to these studies, the value of Ki-67 expression measured by immunocytology as a marker of cellular proliferation has been confirmed in comparative studies with other clinical methods for the

measurement of cell cycle kinetics including ^{3}H-thymidine, simple DNA analysis by flow cytometry, and incorporation of BrdUrd. Again, close correlation with these established techniques was found. These studies of tumors and method comparisons result in conclusive evidence of the association of this antigen with cellular proliferation and support development of its use in multiparameter flow cytometry.

Difficulties in measurement of cell cycle phases would be overcome by the application of multiparameter flow cytometry. In particular, measurement of proliferation-associated antigens simultaneously with DNA analysis would improve evaluation of proliferative activity in tumor cells in mixed cell populations. The measurement of Ki-67 expression in conjunction with DNA staining would also theoretically allow the resolution of G1 cells from G0 cells rather than only determining the S-phase as the proliferation-associated markers BrdUrd and PCNA have done. In addition, the ability to analyze large numbers of cells in a short period of time and the quantitative aspects of flow cytometry are superior to the more cumbersome and subjective technique of traditional microscopic examination of immunocytology preparations.

Preparation and Fixation Procedure

Since there has been no consensus as to the fixation technique most suitable for measurement of Ki-67 expression by flow cytometry, this research had as one of its objectives the determination of the most appropriate technique for Ki-67 measurement. Because the fixation technique desired for Ki-67

measurement in this research needed to produce simultaneously useful DNA staining, the methods utilized for DNA analysis should first be considered. A number of methods exist for propidium iodide (PI) staining and flow cytometric quantitation of cellular DNA content. These methods generally utilize hypotonic solutions (Tate *et al.*, 1983), alcohols (i.e., ethanol or methanol), or detergents (i.e., Triton X-100 or NP-40) to fix and permeabilize cells prior to PI staining. Though the application of alcohol fixation for DNA analysis is well accepted, cell suspensions fixed with alcohols and stained for both nuclear immunofluorescence and DNA content, in general, yield poor immunofluorescence. In addition, fixation with the standard alcohols or detergents often leads to a loss of nuclear antigen localization. In alcohol fixation, these effects are probably caused by a significant alteration of nuclear and cellular architecture due in part to the hypotonicity and dehydrating effects of alcohols.

Another well utilized fixative for flow cytometry that could be relevant to this research was paraformaldehyde. In contrast to alcohols, paraformaldehyde is recognized as one of the best fixatives for cell surface markers and stabilizes cell surface markers even after treatment with 2 N HCl (Houck and Loken, 1985). The action of paraformaldehyde in crosslinking proteins (Puchtler and Meloan, 1985) may serve to stabilize the cell surface markers. But paraformaldehyde fixed cell suspensions yield fluorescence distributions with poor DNA resolution. In paraformaldehyde fixation, these effects are most likely due to crosslinking of the nuclear proteins, thereby altering the interaction of intercalating dyes, such as PI, with DNA.

For these reasons, fixation techniques other than accepted methods have been reported for nuclear antigens. Until recently, flow cytometry has been largely limited to analysis of DNA content or cell surface antigens and has had less application for quantifying nuclear antigens, because of the difficulty involved in introducing antibodies into cells without destroying antigenicity and cell integrity. Still, several methods have been described for localizing nuclear antigens. Most of these methods duplicate staining for DNA content by employing either alcohols (Jacobberger *et al.*, 1986; Kurki *et al.*, 1986; Schutte *et al.*, 1987) or aldehydes combined with a detergent (Clevenger *et al.*, 1985; Czerniak *et al.*, 1987; Mann *et al.*, 1987) to permeabilize cells for accessibility of antibody to the cell interior. Use of these approaches for flow cytometric detection of nuclear antigens have been reported, though these techniques have not gained wide acceptance.

Regardless of the fixation technique utilized, a procedure for simultaneous DNA and nuclear antigen staining must meet the following criteria to be clinically useful: 1) low DNA content coefficient of variation, 2) minimal cellular clumping, 3) well-preserved immunofluorescence staining, and 4) ease and speed of cellular fixation and staining. The fixatives reported in the literature for Ki-67 measurement by flow cytometry: acetone, ethanol, periodate-lysine-paraformaldehyde, and paraformaldehyde/triton/ethanol all failed to meet one or more of these criteria. For this reason, they were judged unsatisfactory for use in this research. Because of the success of the paraformaldehyde/triton/ethanol technique in determining Ki-67 measurements, even though the DNA staining

was unacceptable, evaluation of these reagents using modifications of a procedure reported by Clevenger *et al.* (1985) was continued. After a short fixation in 0.5% paraformaldehyde, fixed cells exposed to a short incubation with 0.1% Triton X-100 showed substantial improvement in DNA staining without an appreciable loss in the detection of Ki-67 expression. Cells fixed in 0.5% PT and stained with PI demonstrated improved fluorescence and lowered CVs in comparison to cells fixed with the other fixatives. Only the 1.0% PT fixed cells had better measurements of Ki-67 expression in cell lines. Because this fixation technique met both the experimental and clinical criteria necessary, it was considered appropriate for use in the second objective of this research.

Expression of Ki-67 in Nonproliferating Cells

An interesting and unexpected finding of this research was the slight change in fluorescence observed between the cells stained with the FITC-labelled irrelevant, isotypic, mouse monoclonal antibody used as a negative control and the fluorescence of nonproliferating cells stained with the FITC-labelled Ki-67 antibody. The mouse monoclonal control utilized is frequently employed in the clinical laboratory for diagnostic flow cytometry procedures and it has been proven to be a reliable reagent. For this reason, it was important to determine if this change was due to an artifactual error resulting from the fixation technique.

To examine this change, a series of experiments were performed. An experiment designed to determine if nonspecific staining of cellular protein by

free FITC was occurring resulted in data supporting the conclusion that this had not taken place. A subsequent experiment showed the results were not due to nonspecific binding of the Ki-67 antibody either.

The use of an indirect staining technique resulted in improvement of the data denoted by a decrease in the Ki-67 fluorescence in nonproliferating cells. Therefore, this approach was utilized for the remainder of the research. Even with the improvement, the excess fluorescence in nonproliferating cells exposed to Ki-67 antibody remained and further investigation continued. An experiment was then performed to titrate both the mouse monoclonal control and the Ki-67 antibody to determine if the results were due to differences in antibody concentration. Since these reagents are produced by different commercial companies, there may be variations in the techniques used to quantitate the amount of immunoglobulin present in each antibody reagent, even though the antibodies were used at the same concentration per test based on the labelled content. Ki-67 antibody titration resulted in better resolution of proliferating and nonproliferating cells. However, mild Ki-67 binding was still detected despite the variation in antibody concentrations. Having eliminated the reagents as a cause of the increased fluorescence in nonproliferating cells, an experiment was carried out to determine if the conditions used for storage of the samples analyzed could be responsible. Expression of the Ki-67 antigen was monitored over a period of 72 hours at 4°C in cell media with differing amounts of newborn calf serum. This was done to rule out low temperature artifacts or serum deprivation as causing an increase in autofluorescence. It was concluded

the refrigeration and serum concentration used for this research were not contributing to the results seen.

As a result of this series of experiments, it was concluded the change in fluorescence between the negative cell populations stained by the mouse control antibody and Ki-67 antibody was due to faint staining of nonproliferating cells. This suggests that contrary to accepted belief, the Ki-67 antigen is expressed at low levels in nonproliferating cells. Expression of the Ki-67 antigen at this low level could make it undetectable by standard techniques in immunocytology, techniques known to be less sensitive than flow cytometry.

Correlation of Flow Cytometry with Immunocytology

Having developed a fixation technique suitable for use with cell lines and normal lymphocytes, a comparison was performed of its effectiveness in measuring Ki-67 expression by flow cytometry in non-Hodgkin's lymphoma versus the measurement of Ki-67 expression by the traditional immunocytology technique in these specimens. The comparison of the two techniques was evaluated by determining the linear correlation between the two measurements. Finding significant correlation between the two techniques was critical because of the wide acceptance of the measurement of Ki-67 expression by immunocytology, both in the literature and in clinical practice. When Ki-67 is measured by immunocytology, pathologists make an empirical estimate of the number of positive stained cells present and report a semi-quantitative approximation of the degree of proliferation present. Adding to the difficulty in the empirical

estimation is the considerable degree of heterogeneity in staining intensity that can be present in a specimen examined. This variation in staining intensity is probably due to differential expression of the antigen at the various phases of the cell cycle. Differing degrees of heterogeneity were observed in the slides examined for this research and this has been observed and reported by other investigators as well (Gerdes *et al.*, 1984a; Hall *et al.*, 1988). The objective quantitation by flow cytometry of the heterogenetic expression of the Ki-67 antigen seen in clinical samples could be of considerable benefit in predicting prognosis or guiding therapeutic decisions. In a comparison performed on twelve non-Hodgkin's lymphoma (NHL) specimens, a significant linear correlation was found (r=0.91), confirming the utility of the developed fixation technique for determination of Ki-67 expression in NHL.

Correlation of Ki-67 and DNA S-Phase in Lymphoma

Having proven the acceptability of the developed fixation technique for quantitation of the expression of the Ki-67 antigen in NHL, a comparison of Ki-67 expression with the measurement of DNA S-phase was performed in these tumors. The comparison of these two techniques was also evaluated by determining the linear correlation between the two measurements. In a comparison performed on 30 NHL biopsies, a significant linear correlation was found (r=0.95) validating this methodology for multiparameter flow cytometry. As observed in the cell lines and normal lymphocytes, an increase in mean fluorescence intensity of Ki-67 labelled nonp. .ferating cells was also detected in

NHL. This observation further supports the suggestion of a low level expression of Ki-67 in G0 cells as evidenced by a faint staining of nonproliferating cells. Also observed in the clinical specimens was a continuum of expression of Ki-67 antigen from the G0 cell population through the G1 cell population. Only in the case of a cell population with a large proliferating compartment was a distinct break observed between the G0 and G1 populations, duplicating the results seen when testing cell lines. Two possible explanations for the continuum seen are: 1) G0 cells have low levels of the Ki-67 antigen expressed, or 2) not all the G1 cells are staining positive for the Ki-67 antigen. Lack of staining of the Ki-67 antigen in G1 cells has been reported as the conclusion for explaining this continuum in two studies (Wersto *et al.*, 1988; Verheijen *et al.*, 1989), but the evidence resulting from this research supports the conclusion of a low level Ki-67 antigen expression in G0 cells.

Correlation of Ki-67 and DNA S-Phase in Non-Lymphoid Tumors

Having found significant correlation between the expression of Ki-67 and the DNA S-phase in lymphomas, the relationship of these two parameters in non-lymphoid tumors was examined. Specimens chosen for evaluation were nine biopsies from breast tumors and eight biopsies from colon tumors. The relationship between the expression of Ki-67 and the DNA S-phase was evaluated by determining the linear correlation between the two measurements. In contrast to the results obtained with lymphoma biopsies, neither the breast tumor biopsies ($r=0.19$) nor the colon tumor biopsies ($r=0.53$) had a significant

correlation between the two parameters. Possible explanations for these results include 1) artifactual errors in cell cycle calculations occurred or 2) the disaggregation procedure utilized resulted in loss of the Ki-67 antigen from the cell nucleus. As the majority of the biopsies consisted of tumor cells in mixed populations of non-neoplastic cells, stromal elements, and/or inflammatory cells, errors in cell cycle calculations could have occurred as a result of the limitations inherent in calculation of the DNA S-phase in mixed cell populations. In addition, more cell debris results from disaggregation of non-lymphoid tumors, thus contributing to the difficulties in calculation of the S-phase fraction. However, a more likely explanation for the lack of significant correlation is leakage of the Ki-67 antigen from the cell nucleus. Since these tumors are difficult to disaggregate into intact single cell suspensions, leakage due to cell membrane rupture could occur. This conclusion is supported by the results of testing for exclusion of trypan blue stain and by cytospin preparations of these biopsies stained by immunocytology techniques. When the non-lymphoid tumor cells were mixed with trypan blue, staining of over 75% of the cells occurred in these tumors, in contrast to the less than 25% staining of the lymphoma tumor cells. Staining of cells with trypan blue reveals cell membrane damage, and the present observations suggest damage of the nuclear membrane may have occurred with subsequent leakage of the Ki-67 antigen possible. Also, in a preliminary examination of immunocytology preparations of non-lymphoid solid tumors, tumor cells were seen displaying cytoplasmic staining by the Ki-67 antibody without nuclear staining. Though cytoplasmic staining by the Ki-67

antibody has not normally been reported in the literature, this may also have contributed to the poor results in these tumors. Further characterization of the Ki-67 antigen and development of improved fixation techniques may be necessary before quantitative flow cytometric analysis of this antigen can be applied universally in solid tumors.

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BIOGRAPHICAL SKETCH

Robert L. Williams graduated in 1977 from the University of Florida, Gainesville, Florida, earning a Bachelor of Health Science degree in medical technology. He was subsequently certified by examination as a medical technologist by the American Society of Clinical Pathologists and as a laboratory technologist by the State of Florida. He then entered the United States Marine Corps Reserve where he was commissioned as a second lieutenant in April of 1978. After serving five years in the logistics career field, he accepted an interservice transfer into the United States Air Force. Following service as a Biomedical Laboratory Officer for six years, he was sent by the Air Force Institute of Technology to earn a Master of Science degree in immunology from the University of Florida. He began graduate studies in the Department of Pathology and Laboratory Medicine in the fall of 1988 and completed a Master of Science degree in medical sciences. He was then assigned as the Chief of Immunology at Brooks Epidemiology Laboratory, at Brooks Air Force Base, San Antonio, Texas, where he plans to continue his career as a clinical immunologist and officer in the United States Air Force.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

Raul C. Braylan, Chair
Professor of Pathology and
Laboratory Medicine

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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and Laboratory Medicine

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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This thesis was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Master of Science.

December 1990

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